On enamel development; Circadian rhythms and Gene expression

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CONTENTS

INTRODUCTION

Enamel, the hardest and most mineralized tissue in the vertebrate body, is formed by ameloblast cells and is incapable of cell-mediated repair or remodelling. Because the circadian clock regulates a broad range of physiological processes, including that of other mineralized tissues influencing bone homeostasis and growth, the periodicity observed in enamel suggests that the molecular underpinnings orchestrating enamel development may be influenced by the circadian clock. Therefore, we wanted to study and investigate whether the microRNAs and their mRNA targets oscillate in a circadian pattern in developing molar tooth germ.

The present work has been carried out at the Department of Oral Biology, Faculty of Dentistry, University of Oslo, during the period 2013-2014. The work consists of two parts:

Part I: This chapter includes basic information concerning molecular genetics of tooth development, enamel formation, structure and growth tracks. It also provides an overview of the biology behind the circadian clock and rhythms. The structure of mature enamel testifies to events that took place during enamel formation. The deposition of enamel is marked by the formation of growth lines, which reflect incremental growth. Gene regulation and molecular mechanisms that are involved during enamel formation resulting in different growth tracks are poorly understood.

Part II: This part is presented as a manuscript entitled: "The Circadian Clock; Expression of mRNAs and microRNAs during enamel development". It deals with the expression of mRNAs and microRNAs during development of molar tooth germ, with special emphasis on the genes coding for enamel matrix proteins and on circadian clock genes.

We contacted associate professor Amer Sehic as we wanted to get an insight into the research field at the faculty. This resulted in a summer research project funded by *The Faculty of Dentistry in Oslo.* We are grateful for the all the help and support from our supervisor associate professor Amer Sehic. We are also grateful for the financial support from the University of Oslo, and want to express our gratitude to the Department of Oral Biology for the opportunity to do this work in an excellent research environment.

Part I

MOLECULAR GENETICS OF TOOTH DEVELOPMENT

The tooth is a convenient experimental model for studying the basic mechanisms of organ development, including differentiation, cellular interaction, morphogenesis, and production and mineralization of extracellular matrices. Tooth development is a continuous process that morphologically can be divided into initiation, bud, cap and bell stages. The mouse dentition consists of one incisor and three molars in each quadrant. Morphologically, murine tooth development begins at about embryonic day 12.5 (E12.5) by thickening of the dental epithelium (initiation stage). The sequence of initiation is incisor (E12.5), first molar (E12.5), second molar (E14.5), and third molar at postnatal day 2 (P2). The epithelial thickening expresses a host of signaling molecules that act to increase cell proliferation at the sites of tooth development. The proliferating epithelium grows further into the underlying neural crest-derived mesenchyme forming a bud. Prior to E12.5, the epithelium supplies information for initiation of a tooth, while after E12.5 the condensing mesenchyme takes over this instructive role. The mouse first molar tooth bud is clearly formed at E13.5, and is enclosed by condensing mesenchyme which produces various signaling molecules (e.g. *Bmp4* and *Msx1*).

Condensation of the dental mesenchyme around the epithelial bud is followed by the induction of a signaling center at the tip of the epithelial bud, the enamel knot. The enamel knot was first described almost 100 years ago, and it is histologically visible as a bump in the center of the inner dental epithelium at the cap stage. This knot of epithelial cells expresses key signaling molecules, such as *Shh*, *Fgf4*, *Bmp4* and *Wnt10b*, and has been considered as an important signaling center for tooth development and tooth morphogenesis. It has been shown that the enamel knots are transient structures, their disappearance by the end of the cap stage being *Bmp4*-dependent. In mice, the incisor tooth germs only have a single enamel knot, whereas molars develop secondary enamel knots. It has been suggested that the primary

enamel knot contributes to the secondary enamel knots. However, this theory is unclear, with fate-mapping experiments and proliferation studies providing both positive and negative evidence. The folding of the inner dental epithelium at the bell stage (E16.5), possibly associated with the secondary enamel knots, results in the formation of a complex multi-cuspid tooth. The deposition of dentin and enamel extends from the epithelium-mesenchymal interface, the outline of which in turn defines the tooth shape. Further, molars develop tertiary enamel knots next to the enamel-free areas at the cusp tips. The size and shape of the primary enamel knot seems to be a clue for the generation of the exact degree of curvature of the oral epithelium. (Fig. 1)

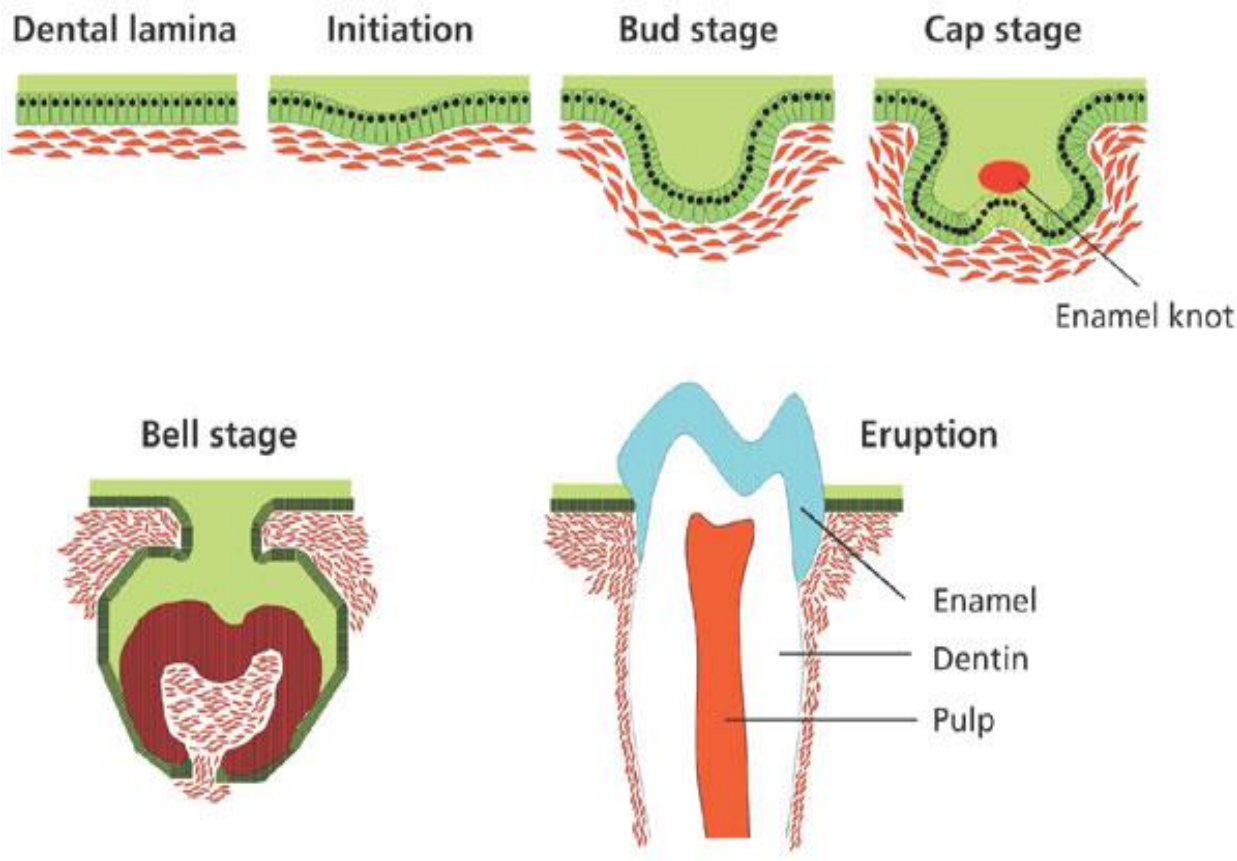

Fig. 1. Developmental stages during tooth development. Reciprocal interaction between ectodermally (green) derived enamel organ and neural crest derived ectomesenchyme (red). At bell stage, dentin-forming odontoblasts and enamel- forming ameloblasts are differentiated (brown) (From Nakashima and Reddi, 2003).

MECHANISMS OF MICRORNA

microRNAs (miRNAs) are a class of non-protein-coding RNAs (~22 nt in length) that is involved in important biological functions, such as development and cell physiology. It has been shown that miRNAs, in a combinatorial fashion, regulate the expression of about 30 percent of protein-coding genes by targeting specific messenger RNAs (mRNAs) for cleavage or translational regulation. In animals, miRNAs often lead to translational repression, and to some extent mRNA decay, through partially complementary pairing to the 3′ untranslated regions (UTRs) of their targets using the seed region (positions 2 to 7 or 8 from 5′ end). However, in plants, miRNAs show perfect complementarity to their targets and lead to mRNA degradation. (Fig. 2)

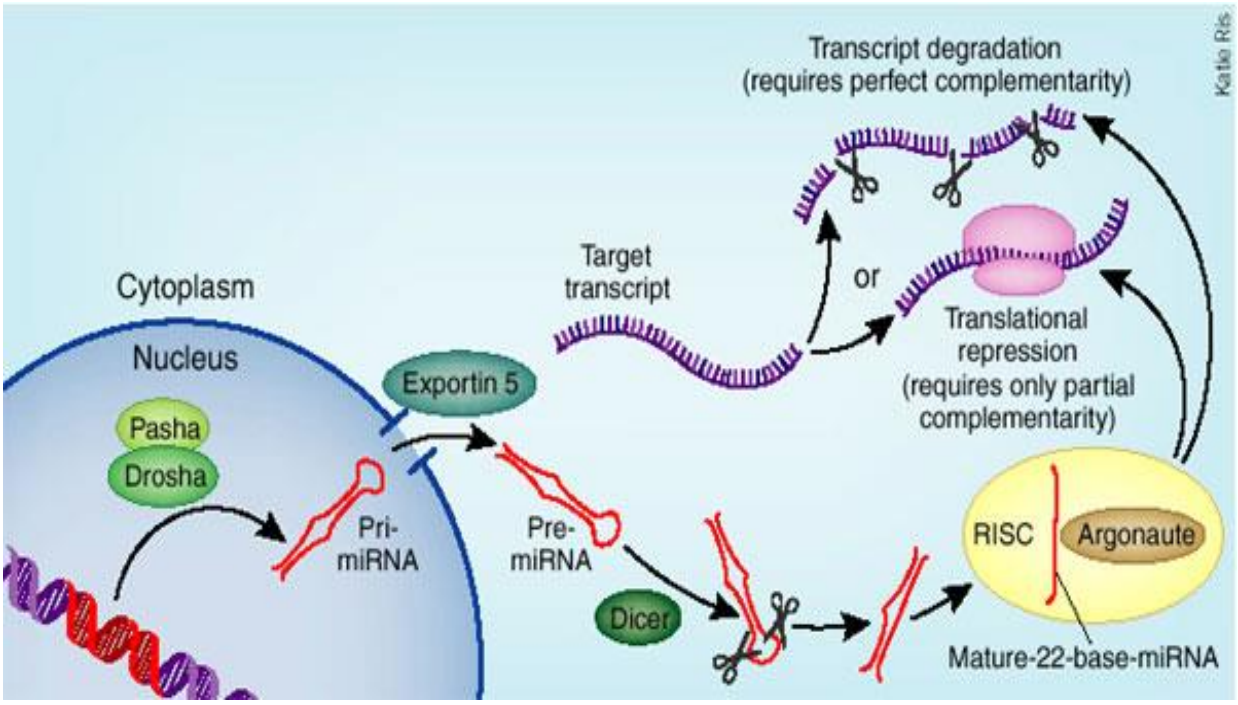

Fig. 2. A primary miRNA (pri-miRNA) transcript is encoded in the cell's DNA and transcribed in the nucleus, processed by an enzyme Drosha and exported into the cytoplasm where it is further processed by Dicer. After strand separation, the mature miRNA represses protein production either by blocking translation or causing transcript degradation (From Mack, 2007).

The longer precursor miRNA molecules are processed in the nucleus into hairpin precursor miRNAs of 70–100 nt by the dsRNA-specific ribonuclease Drosha. The hairpin miRNAs, called pri- miRNA, are transported to the cytoplasm via an exportin-5 dependent mechanism. In the cytoplasm the hairpin miRNA is called pre-miRNA. Here they are digested by a second dsRNA specific ribonuclease called Dicer. The resulting 19–23 nt long mature miRNA is bound by a complex that is similar to the RNA-Induced Silencing Complex (RISC) that participates in RNA interference (RNAi). The mature miRNA leads to translational repression or mRNA degradation, depending on the extent of complementary to the target mRNAs. By a mechanism that is not fully characterized, but which apparently does not usually involve mRNA degradation as in RNAi, the bound mRNA remains untranslated.

miRNAs provide a convenient and efficient pathway for regulation of gene expression at a posttranscriptional level. They exert their effects by base pairing with the target mRNAs in a much more compact and energy-efficient manner compared to regulatory molecules like enzymes and hormones. miRNAs are often organized in tandem and closely clustered on the chromosome. This arrangement can have particular significance in the control of gene expression. Clustered miRNAs with similar sequences may regulate a set of mRNA targets, and these closely related characteristics may allow miRNAs to function as powerful regulators at the cellular level.

In some cases miRNAs have been proposed to act relatively promiscuously and to inhibit multiple targets. Many genes have predicted target sites for several different miRNAs in their 3` UTRs, indicating the possibility for combinatorial action of multiple miRNAs to maximize inhibition of translation. For most mRNAs, however, these predictions have not yet been tested. The prediction of target genes for particular miRNAs is a difficult computational challenge, and is impaired by the lack of data describing alterations in levels of individual protein in response to changes in levels of miRNAs. When more protein data are available, it

will be interesting to compare how miRNA-target interactions correlate with effects on levels of proteins.

Little is known of miRNA function in tooth morphogenesis and differentiation. Studies of miRNA expression profiles of the developing murine molar tooth germ have shown these to be highly dynamic, some miRNAs being abundantly expressed during the early development of the molar tooth germ, while others are highly expressed only at later developmental stages. A recent study also suggested that miRNAs may modulate tooth morphogenesis and ameloblast differentiation, perhaps largely by fine tuning conserved signaling networks. Another study suggested discrete sets of miRNAs to be expressed in molars compared with incisors, as well as in epithelium compared with mesenchyme. Conditional knockout of *Dicer1* in the dental epithelium of the *Pitx2-Cre* mouse results in multiple and branched enamel-free incisors and cuspless molars together with changes in incisor patterning and in incisor and molar size and shape.

In tooth germs miR-214 exhibit a higher level of expression at post-natal stages of odontogenesis, and was therefore in a recent study selected as a target for silencing by *in vivo* transfection of mouse pups with anti-miR-214 in order to study effects on tooth germ transcriptomes and tooth phenotype. The level of miR-214 was about 1/60 of that of the scrambled control 24 h post-transfection with 50 pmol anti-miR. About 1200 mRNAs and 6 miRNAs were found differentially expressed following in vivo transfection with 50 pmol of antagomir-214. Altered levels of mRNAs were also reflected in corresponding changes in levels of the corresponding encoded proteins. Further, the enamel of the resulting mature tooth exhibited evidence of hypo-mineralization.

ENAMEL FORMATION, STRUCTURE AND GROWTH TRACKS

Enamel, the hardest and most mineralized tissue in the vertebrate body, is formed by ameloblast cells and is incapable of cell-mediated repair or remodelling. Tooth enamel is unique among mineralized tissues because of its high mineral content. Enamel is made up of highly organized, tightly packed crystallites, hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, that compromise 87 percent of its volume and 95 percent of its weight. Whereas other mineralized tissues are about 20 percent organic material, mature enamel has less than 1 percent organic matter. Enamel crystallites contain more than one thousand times the volume of corresponding crystals in bone, dentin and cementum. Enamel crystals are extremely long relative to their thickness and highly oriented. They generally extend from the underlying dentin toward the surface of the tooth and are organized into bundles, called prisms. Superior organization and mineralization give dental enamel its outstanding physical properties, making it the hardest tissue in the vertebrate body.

It is easy to deduce that dental enamel formation is under genetic control. The process of enamel formation, or amelogenesis, occurs predictably in tooth after tooth, generation after generation. The size, shape, shade, and even caries susceptibility of dental enamel can be passed from parent to offspring. Genetic diseases are associated with enamel malformations that range from total enamel agenesis to localized defects. Therefore, the formation of dental enamel is somehow encoded in our genes, of DNA. But how can a gene encode a mineral? The answer is that it can not, at least not directly. DNA can only encode RNA, and most of the RNA it encodes is used to make proteins. Dental enamel formation is highly specialized, and the proteins most directly involved in enamel biomineralization are specific for it. As a consequence, defects in the genes encoding enamel proteins generally cause enamel malformations without affecting other parts of the body. There are, however, numerous genetic syndromes associated with dental defects of all types.

Rodent and human enamel exhibits the same basic structural elements, prisms and interprism. However, the spatial arrangement of the prisms, i.e. the prism pattern, is considerably different. In rat and mouse incisors the enamel exhibits two main layers, an inner layer with extreme prism decussation and an outer layer with parallel and incisally inclined prisms. Rat and mouse molar enamel resembles incisor enamel, but prism decussation is absent in some areas. Incremental lines (Retzius lines and prism cross-striations) are not conspicuous in rat and mouse enamel possibly because enamel apposition occurs at a faster rate than in human enamel. Lines resembling incremental lines, with a periodicity of about 1 mm, have been observed in the aprismatic enamel in mouse molars and are probably comparable to laminations. Korvenkontio reported the presence of incremental lines in certain rodent species, but with no conclusive evidence for rat and mouse enamel. Also, lines which are parallel with the surface of developing rat enamel have been induced experimentally by injections of sodium fluoride and tetracycline. Recent study has described the occurrence and periodicity of enamel incremental lines in mouse molars in an attempt to draw attention to some key questions about the rhythm in the activity of the secreting ameloblasts during formation of mouse molar enamel. These lines are probably also comparable to laminations (Retzius-line-like incremental lines with a circadian periodicity). (Fig. 3)

The structural richness of dental enamel is related to the spatial arrangement of the hydroxyapatite crystals. These are not arranged at random; in mammals, the crystals are organized into a pattern of prisms (rods) and interprismatic (interrod) substance. Basically, the prisms are discrete, rod-like entities running from the dentine to the enamel surface, while the interprismatic substance constitutes a continuum in between the prisms. The hydroxyapatite crystals are differently oriented in prisms and interprismatic substance, and this is the sole basis for a distinction between the two; in the prisms, the crystals are oriented with their long axis roughly parallel with the long axis of the prism, while in the interprismatic substance, the

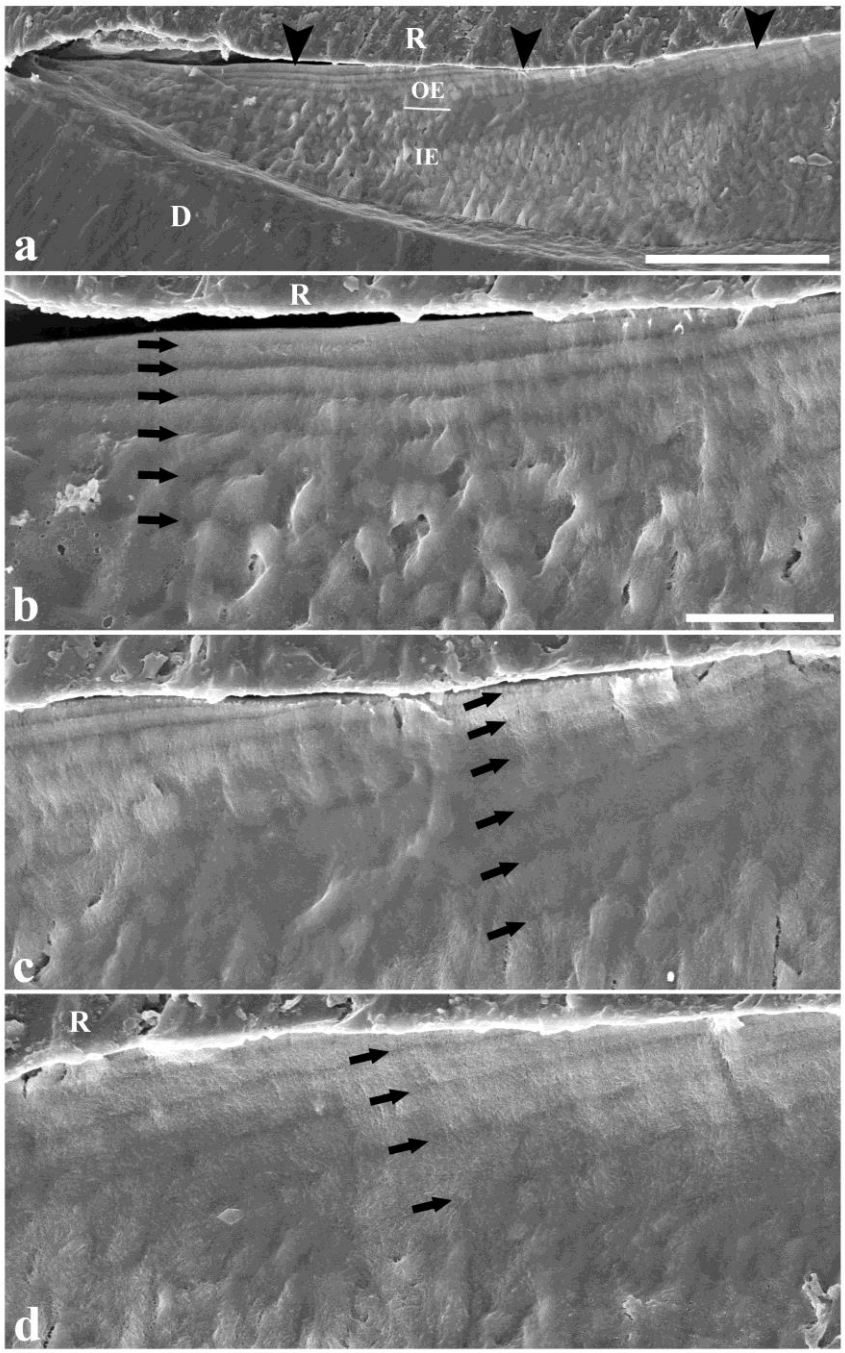

Fig. 3 – SEM images of enamel from distal aspect of second mandibular molar (M2 inf). (b–d) Higher magnification of enamel at the positions indicated by black arrow-heads in panel a. The enamel appears hypomineralized (darker with less distinct crystals and prisms/interprism). The black arrows indicate position and direction of incremental lines in enamel. Incremental lines are seen to proceed some distance into the inner enamel. The periodicity of incremental lines tends to decrease towards to enamel surface. D, dentine; R, resin; OE, outer enamel; IE, inner enamel. The bar represents 50 mm in panel a, and 10 mm in panels b, c and d. (Courtesy of Sehic, Amer et. al., 2013)

crystals tend to be oriented perpendicular to the incremental lines (Retzius lines). At the end of this stage the ameloblasts eventually lose their Tomes`process and produce thin, superficial prism-free enamel. The fine, horizontal grooves on the surface of the crown, the perikymata grooves, represent the external manifestations of the Retzius lines.

The Retzius lines and the prism cross-striations are important structural features related to the growth of enamel. The Retzius lines represent two-dimensional cuts of three-dimensional growth planes, and the basis for their visibility is only partly understood. The regularly spaced Retzius lines prominently present in outer and cervical enamel are thought to represent a 6-10 day rhythm in enamel production. They are structurally characterized by a discontinuity cleft, at least in their outer part, and an increase in interprism at the expense of prisms. The prism cross-striations are thought to represent a daily rhythm in enamel formation. Their visibility has been ascribed to periodic variations in composition along the prisms. Dark bands with reduced concentration of crystals have been observed after acid etching. The cross-striations have been linked to another periodic structural feature occurring along the prisms, the prism varicosities/undulations, but this association has been questioned. Both Retzius lines and cross-striations tend to be accentuated by acid and caries.

Regularly spaced Retzius lines, which indicate a specific rhythm in enamel apposition, could either be caused by an inherent and synchronized rhythm in the activity of the secreting ameloblasts, or by a more general rhythm which also affects ameloblastema. The accentuated Retzius lines, often referred to as pathologic, are probably caused by systemic influences which may range from physiologic fluctuations, as in formation of the neonatal line, to pathologic conditions. This is corroborated by the fact that the pattern of accentuated Retzius lines in teeth within the same dentition is very similar in the enamel that was produced at the same time.

THE CIRCADIAN CLOCK AND RHYTHMS

Due to the fact that the earth circles around the sun, we have a permanent variation in the amount of light that hits us and our planet. We have nights and days, as well as summers and winters. With exception of animals which live in constant darkness, i.e. creatures in the deep oceans or cave dwelling animals, most life on earth depends on the fact that the sun rises every morning, and sets every evening. Because of evolution, some animals live their life at night and sleep during the day, while others, like humans, are active during the day and sleep at night. Life on earth has evolved in a way so that biological processes vary with the daily cycles of night and day. This is called circadian rhythm.

Our retina has light receptors; these help our bodies know whether it is night or day. The light receptors are connected via the retinohypothalamic tract to the suprachiasmatic nucleus (SCN) in the brain. The SCN is believed to be the main hub for controlling the circadian cycles. (Fig. 4a) It is a part of the hypothalamus and works as a circadian pacemaker. With afferent signals from the retina, other parts of the brain and peripheral organs, it regulates the substances that are important for keeping the circadian clock accurate. It has been shown that the SCN has a range of output signals, which fluctuates according to the circadian pattern. Vasopressin was the first substance to be discovered; later researchers also found that vasoactive intestinal polypeptide was secreted from cells in the SCN in a circadian manner. The receiver of the suprachiasmatic nucleus' outflow of information is the medial hypothalamus. The SCN neurons containing vasopressin has endings near corticotrophin-releasing hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus (PVN). CRH is the hormone responsible for the release of adrenocorticotrophic hormone, which signals the adrenal glands to make them release corticosterone. In a research done on both nocturnal and diurnal rats, scientists found that vasopressin has different effects on the two. Both animals expressed vasopressin during the light period of the day. In nocturnal rats

vasopressin inhibited the secretion of CRH, while in diurnal rats it stimulated the CRH containing neurons. The SCN is connected to the pineal gland via the PVN. The pineal gland excretes melatonin, which is an important hormone during the dark hours of the circadian cycle. Glutamatergic and GABAergic neurons run from the SCN to the PVN. The glutamatergic neurons show a continuous excitation of the sympathetic pre-autonomic neurons in the PVN. During the day, the neurons in the PVN are inhibited by GABAergic neurons. The GABAergic neurons activity behaves in a circadian matter, and the GABAergic effects on the PVN are reduced during the night. This excites the output neurons from the PVN, giving the pineal gland a signal to release melatonin during the dark hours. The SCN is believed to influence the daily cycles of many hormones, including luteinizing hormone, plasma thyroid hormone and glucoregulatory hormones. In addition it will also work through the autonomous nervous system to influence the sensitivity to hormonal signals on the target tissues. Interestingly, the SCN can act autonomously. This means that it can regulate the circadian cycle without external cues. Studies have shown that when an individual is exposed to continuous darkness, the SCNs rhythmic activity can persist for weeks, months and even years. However, any external cue will help entrain the SCN to synchronize with the outside world.

External cues for setting the circadian clock are called *Zeitgeber*, the most important of these being the light-dark cycle. For animals living in the Arctic, where the sun may never set, it has been shown that light intensity and temperature can impact the synchronization of the circadian clock. When the temperature falls in the Arctic night, invertebrate food is harder to find, helping animals here entrain their circadian clock.

Clock genes are genes that regulate and maintain the circadian clock function; this is done in a complicated matter. This explanation will feature the essential steps of gene and protein regulation. At the top of this hierarchical system are the clock-genes and -proteins

Bmal1 and CLOCK. These are transcription factors, and they cause the transcription of other genes which in turn, via a negative feedback loop, inhibit CLOCK and Bmal1. CLOCK and Bmal1 will form a heterodimer and bind DNA of clock target genes at their E-box response elements. The CLOCK/Bmal1 heterodimer will activate transcription of the other clock-genes *Per, Cry,* and *Rev-erbα.* The clock proteins Per and Cry are made, and they translocate to the cytoplasm where they dimerize. This Per/Cry-dimer will translocate back to the nucleus where it will inhibit the CLOCK/Bmal1-complex from initiating transcription. Rev-erbα inhibits transcription of Bmal1 mRNA. Since the Per/Cry- dimer inhibit CLOCK/Bmal1, it will essentially be the activator of the transcription of Bmal1 mRNA, since transcription of Bmal1 happens in the absence of Rev- erbα. (Fig. 4b)

It is suggested that life in its early stage developed ways to distinguish night from day to protect itself during DNA-synthesis. UV-radiation is harmful to DNA, thus making it an advantage to execute DNA-synthesis at night. The expressions of a number of genes are also varying in 24 hour cycles. Many hormones, like melatonin, show a circadian pattern and evidence show that they may account for the fact that we feel tired and sleepy when the sun is down and alert and rested when the sun is up.

In addition to external cues and the SCNs autonome regulation, research has shown that microRNA can regulate expression of genes in a circadian manner. However, few microRNAs and target clock genes have been reported in the circadian literature, especially what regards tooth development.

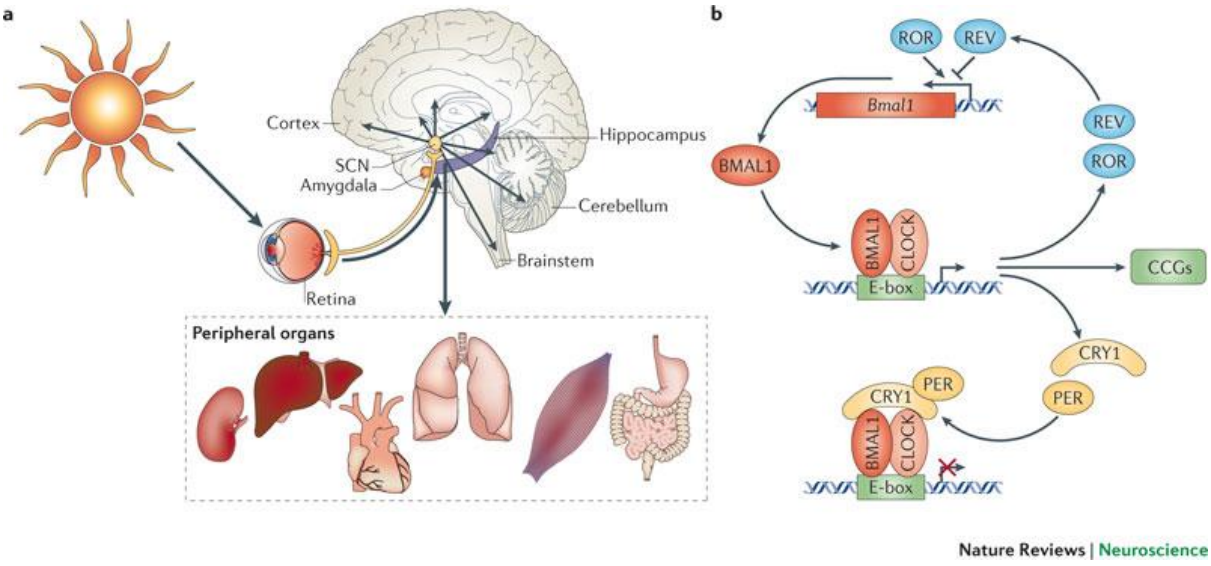

Fig. 4. a | A hierarchical organization of the circadian clock. The master pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus and generates internal circadian rhythms in gene expression, electrophysiology and hormone secretion. Direct projection from the retina transfers information about light and darkness to the SCN, which synchronizes a phase of SCN rhythms with the external environment. Local circadian clocks are found in different brain regions and throughout the body (peripheral oscillators). Electrical and humoral signals from the SCN synchronize phases of local circadian clocks oscillations. Local circadian clocks generate rhythms in gene expression, metabolism and other physiological activities. **b** | The molecular circadian oscillator. For the circadian clock transcriptional–translational negative feedback loop, positive elements are shown in red, negative elements in yellow and components that stabilize the loop are shown in blue. The basic-helix–loop–helix-PAS domain-containing transcription factors BMAL1 (brain and muscle ARNT-like 1) and CLOCK (circadian locomotor output cycles kaput) — or NPAS2 (neuronal PAS domain-containing protein 2) — regulate the transcription of genes with circadian E-box elements in the promoter. These transcription factors also represent positive elements of the transcriptional–translational feedback loop. The BMAL1–CLOCK complex activates the expression of period (*Per*) and cryptochrome (*Cry*) genes. PER and CRY represent negative elements of the loop; they form complexes and inhibit the activity of BMAL1–CLOCK and hence their own expression. Rev-Erbα and retinoic orphan receptors (RORs) represent an additional loop; Rev-Erbα negatively regulates the expression of BMAL1, whereas RORs positively regulate the expression of BMAL1. Finally, the BMAL1–CLOCK complex regulates the expression of circadian-clock-controlled genes (CCGs), which provide a circadian output in physiology (From Kondratova and Kondratov 2012).

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Part II

The Circadian Clock; Expression of mRNAs and microRNAs during enamel development

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A running title: *Circadian controls during amelogenesis*

Abstract

Ameloblasts, the cells making dental enamel, modify their morphological features in response to specialized functions necessary for synchronized ameloblast differentiation and enamel formation. Secretory and maturation ameloblasts are characterized by the expression of stage-specific genes which follow strictly controlled repetitive patterns. The daily incremental growth of enamel has led to the hypothesis that the circadian clock may be involved in the regulation of enamel development. Circadian rhythms and expression of genes in the tooth germ, including enamel-related genes, and microRNAs have not yet been thoroughly described and understood. The aim of this study was to analyze and investigate whether the microRNAs and their mRNA targets oscillate in a circadian pattern in developing molar tooth germ. Comparative gene-expression profiling was carried out on RNA isolated from molar tooth germs from the mice that were sacrificed in the morning, after being exposed to dark for 12 hours, and from molar tooth germs from the mice that were sacrificed in the evening, after being exposed to light for 12 hours. Validation of expression data was achieved using real-time reverse transcription-polymerase chain reaction (RT-PCR). Our data shows evidence that the main clock genes (*Per1, Per2, Clock and Bmal1*) oscillate in mouse molar tooth germ at RNA level. Genes coding for enamel matrix proteins (*Amelx, Ambn and Enam*) also exhibited oscillatory expression pattern. Taken together, our data suggest that changes in clock gene expression patterns may result in significant alterations if enamel apposition and mineralization. Further, our results support the hypothesis that the circadian clock temporally regulates enamel development.

Keywords: Dental enamel; Molar; Mouse; Microarrays; Ameloblast

Introduction

Enamel, the hardest and most mineralized tissue in the vertebrate body, is formed by ameloblast cells and is incapable of cell-mediated repair or remodelling. Enamel formation starts at the enamel-dentin junction and proceeds outward by layered apposition of the matrix produced and secreted by the retreating ameloblasts. This movement of ameloblasts brings them from the enamel-dentin junction to the surface of the enamel. The path pursued by each individual ameloblast is traced out by the prisms, while the movement of the ameloblast layer as a whole is mirrored by the incremental lines of enamel, the Retzius lines (Schour and Massler 1940). These lines, therefore, indicate the position of the ameloblast layer and of the developing enamel surface at different points of time and may evidence physiological or pathological events affecting enamel formation. A system of evenly spaced incremental lines, especially evident in the outer and cervical enamel of humans and other primates has been thoroughly described, and is highly indicative of a physiological rhythm in enamel formation (Fukuhara 1959; Gustafson 1959; Risnes 1985, 1990, 1998). Shorter increments, the prism cross-striations, probably represent a diurnal rhythm in enamel formation (Schour and Poncher 1937; Bromage 1991; Risnes 1986). The number of prism cross-striations between any two regularly spaced Retzius lines is reported to vary between 6 and 11 (Risnes 1998). These incremental growth tracks, Retzius lines and prism cross-striations, represent an internal record of time which, linked to a time scale, may serve as a valuable tool in determining the rate of enamel formation (Risnes 1986) and the crown formation time (Dean and Scandrett 1996). In ungulate enamel incremental lines (called laminations) with a supposedly circadian periodicity comparable to prism cross-striations, but of a continuous nature comparable to and parallel with the Retzius lines, have been described (Kierdorf et al. 2012, 2013). In human enamel continuous lines with a periodicity comparable to and in register with prism cross-striations have been observed in aprismatic enamel (Risnes 1998; Li

and Risnes 2004). Also, sub-daily (intradian) incremental lines have been described both in ungulate (Kierdorf et al. 2012, 2013) and primate (Smith et al. 2003, 2004) enamel.

Because the circadian clock regulates a broad range of physiological processes (King and Takahashi 2000; Reppert and Weaver 2002; Schibler and Sassone-Corsi 2002; DeBruyne et al. 2007), including that of other mineralized tissues influencing bone homeostasis and growth (Fu et al. 2005; Zvonic et al. 2007; Gafni et al. 2009), the periodicity observed in enamel suggests that the molecular underpinnings orchestrating enamel development may be influenced by the circadian clock (Boyde 1989, 1990). It is known that ablation of the suprachiasmatic nucleus in rats results in a disrupted patterning of incremental lines in dentine (Andresen lines), supporting the involvement of the circadian clock in the formation of this tissue (Ohtsuka-Isoya et al. 2001). However, the association between the circadian clock and enamel development remains poorly defined. Immunohistochemical (IHC) studies have shown the expression of *Clock, Bmal1, Per1,* and *Per2* proteins in the developing tooth organ (Zheng et al. 2011). Moreover, intravenous injections of 3H-methionine administered at different times of the day visualized daily variations of radiolabeled proteins secreted by ameloblasts (Smith and Nanci 1996; Xu et al. 2006; Simmer et al. 2010). Using in situ hybridization data and transcriptional analysis of the amelogenin promoter in mice, suggested a cyclic production pattern of amelogenin. By using an ameloblast cell line, other researchers have explored the potential links between circadian control and stage-specific regulation of ameloblast genes (Athanassiou-Papaethymiou et al. 2011). Another recent study has demonstrated that circadian clock genes in an ameloblast cell line and amelogenin gene (*Amelx*) in 2-day postnatal mouse molars oscillate in a circadian pattern (Lacruz et al. 2012). These studies suggest that circadian clock genes modulate enamel development, and that amelogenesis is subject to diurnal rhythms in gene expression levels and cell activity during development of mouse molar enamel. Nevertheless, circadian rhythms and expression of other

genes in the tooth germ, including enamel-related genes, and microRNAs have not yet been described. The recent report brought new insights into possible roles of microRNAs in modulating circadian clocks, revealing that the interplay between different types of RNAs and proteins can exert modulation of circadian-clock period and entrainment (Chen et al. 2013). Therefore, we wanted to analyze and investigate whether the microRNAs and their mRNA targets oscillate in a circadian pattern in developing molar tooth germ.

Materials and methods

Experimental animals

All animals were kept at a 12h light:dark cycle, 21^{o}C with a relative humidity of 65%. Prior to experimental use, animals were given standard laboratory fodder and water *ad libitum*. The animals were kept according to the regulations of the Norwegian Gene Technology Act of 1994. The right mandibular molar tooth germ was isolated from mouse newborns (CD-1 strain) at postnatal day 1 (P1). The first group of the molar tooth germs (P1-morning) was isolated from the mice that were sacrificed in the morning, after being exposed to dark for 12 hours. The other group of the molar tooth germs (P1-evening) was isolated from the mice that were sacrificed in the evening, after being exposed to light for 12 hours. The day of appearance of a vaginal plug was set to 0.5 days post coitum and pups were born at P0. Embryos were staged according to the Theiler criteria (Kaufman 1994). The newborns were sacrificed by decapitation and their heads were immediately immersed in RNAlater (Ambion, Austin, TX, USA). The right mandibular molar tooth germ was dissected out while immersed in RNAlater diluted 1:4 with phosphate-buffered saline (PBS) and subsequently transferred into undiluted RNAlater.

Isolation of RNA

Fractions of total RNA, and RNA-fractions enriched with respect to miRNAs, were isolated according to the manufacturer`s protocol from the molar tooth germs using the Qiagen RNA Mini-kit and Qiagen miRNAeasy Mini-kit respectively (Qiagen, Hilden, Germany). This yielded RNA fractions exhibiting a ratio of OD_{260}/OD_{280} and OD_{260}/OD_{230} of at least 1.8 and 2.0, respectively. RNA was isolated from the P1-morning tooth germs and from the P1-evening tooth germs from at least ten phenotypical newborns.

The quality of RNA in solutions was assessed using Agilent-Bioanalyzer (Agilent, Palo Alto, CA, USA). All solutions used had RIN (RNA integrity number) values higher than 8.5. Concentrations of solutions of purified RNA were assayed using Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Microarray analysis of mRNAs

Mouse OneArray microarrays (Phalanx Biotech Group, Palo Alto, CA, USA) used in this study included a series of control spots to monitor the quality of sample processing, hybridization efficiency, and the specificity of hybridization. Microarray analysis was carried out in duplicates for both groups; each of two microarrays was hybridised with cDNA prepared from 1µg of RNA derived from each of the resulting solutions of RNA. cDNA from P1-morning tooth germ was Cy5 labelled while cDNA from P1-evening was Cy3 labelled.

Preparation of cDNA, Cy3- and Cy5- labelling and hybridization were carried out using the Genisphere 3DNA Array 900 detection kit as described by the manufacturer (Genisphere, Hatfield, PA, USA). Hybridization of labelled samples of RNA was carried out at 58 ^{o}C for 18h in a SlideBooster 400 Hybridization Station (Advalytix, Munich, Germany). The microarrays were scanned in a Packard Bioscience Scanarray Lite microarray scanner (Perkin-Elmer, Waltham, MA, USA). The Cy3 and Cy5 fluorescence signals were quantified by using the SCANARRAY EXPRESS v. 3.0 program (Perkin-Elmer). The resulting fluorescence data (contained in .csv-files) was analysed using the SPOTFIRE v. 9.0 microarray analysis program (Spotfire, Somerville, MA, USA). The microarray methods were otherwise as described previously (Sehic et al. 2009).

Microarray analysis of miRNAs

Mouse & Rat miRNA OneArray microarrays (Phalanx Biotech Group, Palo Alto, CA, USA) were used. The array probe set included 728 unique miRNA sequences common to mice and rats. The arrays also included 135 control spots derived from 25 unique sequences which were

drawn from a variety of organisms to act as positive and negative controls. All sequences had been printed in triplicates. The miRNA-enriched fractions were labelled using Kreatech ULS labelling of miRNA (Kreatech, Amsterdam, Netherlands). Each slide was hybridized with samples containing 1µg of RNA. Hybridization of labelled samples of RNA was carried out at 37 ^{o}C for 16h in a SlideBooster 400 Hybridization Station (Advalytix). Microarray analysis was carried out in duplicates with samples from molar tooth germs from both groups. The microarray methods used were otherwise as described above.

Real-time RT-PCR assays

Levels of expression of miR-210, miR-214, miR-466h and miR-145 in first mandibular molar tooth germ were measured by real-time RT-PCR using the TaqMan MicroRNA kit protocol and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA).

Expression of selected genes (*Ambn, Amelx, Enam, Bmal1, Clock, Per1 and Per2)* were assayed by real-time quantitative RT-PCR using primers designed with Primer3. cDNA was synthesized by oligo-dT priming using a First Strand Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany). Real-time PCR assays were carried out with a qPCR core kit (Eurogentec, Seraing, Belgium).

Total RNA isolated from five molar tooth germs from both P1-morning and P1-evening mice was used for RT-PCR assays. Every analysis was carried out in technical and biological triplicates. Statistical evaluation of the significance of differences between measured cycle threshold (Ct) values was carried out using the REST 2005 program (Pfaffl et al. 2002). All RT-PCR assays were carried out using the Stratagene Mx 3005P PCR instrument (Stratagene, LaJolla, CA, USA).

Statistical analysis of microarray data

Statistical analysis of mRNA microarray data was carried out on data derived from sets of duplicate slides which were combined into a single data-file. Genes exhibiting net fluorescence values of less than 300 in both channels were not included in further analysis. False discovery rate (FDR) of Benjamini et al. 2001 was used to correct selection of genes for false positives ($P<0.05$). The ANOVA facility of the SPOTFIRE program was used to select genes which exhibited statistically significant differences in levels of expression ($P<0.05$) in the molar tooth germ isolated at P1-morning compared with molar tooth germ isolated at P1-evening.

Statistical analysis of miRNA microarray data was carried out on data derived from sets of duplicate slides which were combined into a single data-file. miRNAs exhibiting net fluorescence values of less than 500 in both channels from two out of three replicate spots were not included in further analysis. The statistical analysis of microarray data was otherwise as described previously (Sehic et al. 2009).

Bioinformatic analysis

Bioinformatic analysis using Ingenuity Pathway Analysis (IPA) was carried out in an attempt to provide molecular and cellular functional interpretations of genes exhibiting significantly different expression ($P<0.05$) in the molar tooth germ isolated at P1-morning compared with molar tooth germ isolated at P1-evening.

Bioinformatic analysis of miRNA expression data is relatively unexplored compared with bioinformatic analysis of mRNA expression data. In this study we used TargetScan Release 5.2 software (http://www.targetscan.org) in combination with IPA in order to identify computationally predicted mRNA targets for miRNAs which were differentially expressed in tooth germs of newborns in P1-morning group compared with tooth germs of newborns in P1-evening group.

Results

Microarray analysis of differentially expressed mRNAs and miRNAs

Using the ANOVA function within the Spotfire program it was possible to screen the entire data-set for genes which showed significantly different ($P<0.05$) levels of expression in P1-morning tooth germ compared with P1-evening tooth germ. This yielded 439 genes (Fig. 1). Among these differentially expressed genes 228 exhibited lower level of expression in the morning after being in the dark period for 12 hours, while 211 genes exhibited higher levels of expression after the dark period. Further, the 439 mRNAs that exhibited different levels of expression were organized by self-organizing maps (SOM) using the Spotfire program (Fig. 2). Based on the pattern of expression of these genes, they could be divided into 5 groups (Fig. 2). The 228 genes that exhibited lower levels of expression in the morning were clustered into 3 groups (SOM 1 with 39 genes, SOM 2 with 153 genes and SOM 3 with 36 genes) (Fig. 2). The 211 genes that exhibited higher levels of expression in the morning were clustered into 2 groups (SOM 1 with 79 genes and SOM 2 with 132 genes) (Fig. 2).

Expression of miRNAs in the developing molar tooth germ

Differentially expressed miRNAs in tooth germs were identified using ANOVA. Microarray analysis of miRNAs suggested that 32 miRNAs exhibited significantly different levels of expression in the P1-morning molar tooth germ compared with P1-evening molar tooth germ. These 32 miRNAs detected in the molar tooth germ were hierarchically clustered (Fig. 3). Based on the pattern of expression of these miRNAs they could be divided into two groups. The 12 miRNAs in group 1 exhibited lower levels of expression in P1-morning compared with P1-evening tooth germ. Similarly, the 20 miRNAs in group 2 exhibited higher levels of expression in P1-morning compared with P1-evening tooth germ (Fig. 3).

Validation of microarray analysis by real-time RT-PCR

To validate microarray data, seven genes (*Ambn*, *Amelx*, *Enam*, *Bmal1*, *Clock*, *Per1* and *Per2*) were selected to have their expression assayed also by real-time RT-PCR. These genes were selected because, based on microarray data, their patterns of expression were different between P1-morning and P1-evening tooth germs. Results obtained using real-time RT-PCR show good correlation with results obtained with microarrays (Fig. 4A). Three of the selected mRNAs exhibited lower levels of expression in the morning, after being exposed to dark for 12 hours, i.e. *Ambn* (coding for ameloblastin), *Amelx* (coding for amelogenin) and *Enam* (coding for enamelin), most markedly *Amelx* and *Ambn* (Fig. 4A). Four of the selected genes showed increased expression in the morning, i.e. *Clock* (coding for circadian locomotor output cycles kaput), *Bmal1* (coding for aryl hydrocarbon receptor nuclear translocator-like) and *Per2* (coding for period circadian protein homolog 2). The level of expression of *Per1* (coding for period circadian protein homolog 1) was not significantly different between the morning and evening tooth germ.

Levels of selected miRNAs (mmu-miR-210, mmu-miR-145, mmu-miR-214 and mmu-miR-466h) in P1-morning and P1-evening tooth germ were also measured by quantitative real-time RT-PCR. The microarray data suggested that their levels of expression differed between the two experimental groups (Fig. 3). Expression of mmu-miR-145 and mmu-miR-466h exhibited lower levels of expression after a dark period, while the expression of mmu-miR-214 and mmu-miR-210 exhibited higher levels of expression after a dark period (Fig. 4B). The RT-PCR results showed good agreement with microarray results (Fig. 3, Fig. 4B).

Molecular and cellular functions with significant associations with genes exhibiting significantly *lower* levels of expression at P1-morning, after the dark period

Ingenuity Pathway Analysis (IPA) recognized every gene that exhibited lower levels of expression in P1-morning compared with P1-evening tooth germ. This constitutes 39, 153 and 36 genes in SOM 1, 2 and 3, respectively. Bioinformatic analysis of these genes should provide a guide to molecular and cellular functions which likely are down-regulated in tooth germ after a dark period. IPA suggested significant ($P<0.05$) associations between these genes and various molecular and cellular functions (Fig. 5). Results presented in Fig. 5 show that several categories were highly associated with these genes, e.g. “Cellular movement”, “Tissue morphology”, “Cell morphology”, “Cell cycle”, “Dental disease”, “Lipid metabolism” and “Cell morphology”.

Molecular and cellular functions with significant associations with genes exhibiting significantly *higher* levels of expression at P1-morning, after the dark period

The 211 genes exhibited significantly increased levels of expression in P1-morning compared with P1-evening tooth germ, and constituted 79 and 132 genes in SOM 1 and 2, respectively. Results presented in Fig. 6 show molecular and cellular functions found to be significantly associated with populations of these genes, e.g. “Cell cycle”, “Gene expression”, “Cellular development”, “Cell death and proliferation” and “Cellular growth and proliferation”. These associated cellular functions may therefor exhibit higher activities in the tooth germ after being exposed to dark for several hours.

Predicted mRNA targets and differentially expressed miRNAs

Using TargetScan and Ingenuity Pathway Analysis we found that 32 miRNAs that exhibited different levels of expression in P1-morning tooth germ compared to P1-evening tooth germ (Fig.3) had several predicted mRNA targets. Some of these targets were also found among differentially expressed mRNAs (Fig. 1 and 2), and are presented in Table 1. A major fraction

(28 miRNAs) of these miRNAs had predicted targets that were found in our data of differentially expressed genes, either with lower or higher expression in P1-morning tooth germ. The remaining four miRNAs (miR-466h, miR-466i, miR-471 and miR-805) did not have any predicted targets that were differentially expressed and found in our data (Table 1).

On the other hand Table 2 presents 37 genes from the group that exhibited lower levels of expression in P1-morning tooth germ and their associated miRNAs. Some of these genes (e.g. *Ambn, Fam20a, Enam*) are targeted by several miRNAs, while some are not known to be targeted by any of the miRNAs we found to be differentially expressed in our data. Several of these genes are highly linked to tooth development and enamel formation, i.e. *Amelx, Ambn, Amtn, Clu* and *Enam*.

Discussion

Circadian rhythms are self-sustained endogenous oscillations that occur over a 24-h period. They correspond to the environmental light–dark cycles of an organism but persist even after the light–dark stimulus has been removed. These biological rhythms are involved in most physiological processes. Although there is a site in the suprachiasmatic nucleus of the brain that is considered as the "master clock", peripheral clocks have been found in several tissues in the body. The relationship between these two types of circadian biological clocks is, as yet, unclear (Brown and Schibler 1999). Several genes have been identified as core maintainers of the circadian rhythm. The main mammalian genes include Circadian Locomotor Output Cycles Kaput (Clock), Brain and Muscle Aryl Hydrocarbon Receptor Nuclear Translocation (ARNT)-like (Bmal1), Period 1 (Per1), Period 2 (Per2), Period 3 (Per3), Cryptochromes (Cry1 and Cry2*)*. The genes Nr1d1, Nr1d2, RAR-Related Orphan Receptor Alpha (Rora), and Albumin D-binding protein (Dbp) also play a key role in modifying the expression of the main clock genes (Ukai-Tadenuma et al. 2008). Transcription of these clock genes oscillates

over a 24-h period and their output signals induce rhythms of target gene expression that create patterns in physiological processes. Inducing a rhythm involves the binding of a clock gene transcription factor to the promoter region of a clock-controlled gene (Jin et al. 1996).

We have investigated some aspects of the possible association between the circadian clock and tooth and enamel development. The most striking findings emerging from our results is that numerous genes exhibit significantly different levels of expression in mouse molar tooth germ during the dark period compared with the light period. The other interesting finding is that among these differentially expressed genes we found genes essential for enamel formation by coding for enamel matrix proteins amelogenin, ameloblastin and enamelin. Validation of microarray data using RT-PCR suggested good correlation between the two methods as judged from results obtained with 7 genes and 4 miRNAs (Fig. 4).

It has recently been shown that clock genes and clock proteins are expressed during ameloblast differentiation (Simmer et al. 2010, Zheng et al 2011). The present study using mouse molar tooth germ further supports the concept that clock genes are expressed in ameloblasts and that their expression oscillates during 24-h intervals. We have shown that the circadian clock genes, especially *Clock*, *Bmal1* and *Per2* (Fig. 4A) oscillate in mandibular mouse molar tooth germ over 24 hours. Importantly, we also detected daily oscillations in the expression of *Amelx* in the ameloblasts of whole developing mouse molars. This is in accordance with previous study (Lacruz et al. 2012). *Amelx* is a differentiation-specific product of ameloblast cells. The expression of *Amelx* decreased markedly during the dark period relative to the light period. However, *Amelx* expression does not cease over the sampled periods but instead oscillates, as previously proposed (Boyde 1990). These data confirm the histological analysis of mineralized enamel in which fast- and slow-growth periods were observed over 24 hours (Boyde 1989) and also confirm previous in situ hybridization data showing changes in *Amelx* expression in whole mouse incisors (Xu et al.

2006). Our data also suggest that the expression of genes associated with other functions in the developing tooth such as insulin-like growth factor 2 (*Igf2*) and troponin T (*Tnnt*) increases expression during the night, when *Amelx* is low. *Igf2* has previously been found to be expressed at prenatal stages of tooth development, while *Tnnt* is showed to be expressed in molar tooth germ and is associated with actin cytoskeleton signaling (Sehic et al. 2009, Khan et al. 2012). These results suggest that ameloblasts segregate these functions according to the time of the day, with a possible critical dependency on *Amelx* production or reduction during cycles of fabrication. Our data suggest that other enamel specific genes (*Ambn* and *Enam*) are also likely influenced by the circadian clock (Fig. 4A). Furthermore, as shown in this study several other mRNAs of enamel- and nonenamel-specific gene products and associated molecular and cellular functions changes between day and night, suggesting that some functions may therefor exhibit different activities in the tooth germ during the dark compared with during the day light. Bioinformatic analysis suggested highly significant associations between differentially expressed genes and cellular functions relating to cell division and cell growth (Fig. 5 and 6). Most of the genes associated with these cellular functions were down-regulated in the morning, after being exposed to dark for 12 hours.

miRNAs provide a convenient and efficient pathway for regulation of gene expression at a posttranscriptional level. miRNAs exert their effects by base pairing with the target by binding to specific messenger RNAs (mRNAs) for cleavage or translational regulation. In general, when the specific miRNA is highly expressed, its target mRNA should exhibit low levels of expression. However, it has previously been shown that altered cellular levels of miRNAs may also increase levels of expression of mRNAs (Krutzfeldt et al. 2005, Sehic et al. 2011). However, the mechanism for this is unknown. In our data we found both up-regulated and down-regulated mRNAs, having their expression at the same level/direction as their miRNA. However, we have also observed the opposite which is more expected, e.g. highly

expressed miRNAs at P1-morning (e.g. miR-466g and miR-466f-3p) and their respective mRNA targets (*Amelx* and *Ambn*) which exhibit low levels of expression at P1-morning. These results suggest that at least some of the miRNA and their mRNA targets oscillate in molar tooth germ, which based on these findings also strength their prediction-hypothesis, i.e. *Amelx* and *Ambn* seem to be targeted by miR-466g and miR-466f-3p, respectively.

Taken together, our results suggest that clock genes regulate several ameloblast stage-specific genes, supporting the idea that clock genes are key regulators of ameloblast differentiation. These data are consistent with previous discoveries reporting that the amounts of AMELX secreted vary during different daily intervals (Simmer et al. 2010). More studies are needed to understand the precise roles of clock genes in enamel formation. Nevertheless, we suggest that in addition to the stage-specific controls, amelogenesis is subject to very precise, rhythmic daily controls of gene-expression levels and cell activity.

In conclusion, our study offers some insights into the role of clock genes in ameloblast differentiation and explores the potential links between circadian control and stage-specific regulation of ameloblast genes. The hypothesis, that an ameloblast peripheral clock regulates enamel formation orchestrating the expression of ameloblast-specific genes, is further strengthened. Direct links between changes in the expression of clock genes and dental diseases remain to be confirmed using in vivo models. Nevertheless, this initial study, using whole molar tooth germ, lays the foundation for more research in the chronobiology of tooth development and diseases. Further, it would be very interesting to study the expression of clock genes and genes coding for enamel matrix protein at several time-points during the day/night. The 12 hour span, as used in this study, is quite long interval, and may result in lost information that probably is important.

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Legends to Figures

Figure 1. Genes (mRNAs) exhibiting differential expression in the mandibular first molar tooth germ

Two groups, each of ten newborn pups, were sacrificed and molar tooth germs were dissected out and used to isolate total RNA fractions as described in Materials & Methods. The first group of the molar tooth germs (P1-morning) was isolated from the mice that were sacrificed in the morning, after being exposed to dark for 12 hours. The other group of the molar tooth germs (P1-evening) was isolated from the mice that were sacrificed in the evening, after being exposed to light for 12 hours. Expression profiling of mRNAs was carried out using microarrays, and differentially expressed ($P<0.05$) mRNAs were isolated using the analysis of variance (ANOVA) function in the SPOTFIRE program. Microarray analysis was carried out in technical duplicates.

Figure 2. Self-Organizing Maps (SOM) clustering of differentially expressed genes

Differences in gene expression in tooth germs isolated at P1-morning and P1-evening are here visualized by SOM clustering. In order to isolate a population of genes that may be functionally related to each other, the expression data were organized into 9 clusters using Spotfire program. The results show that 228 genes that exhibited lower levels of expression in the tooth germs from P1-morning were clustered in to three groups (SOM 1, 2 and 3). The remaining 211 genes that exhibited higher levels of expression in the tooth germs from P1-morning were clustered in to two groups (SOM 1 and 2).

Figure 3. Heat map resulting from hierarchical clustering of the 32 miRNAs exhibiting differential expression in the first mandibular P1-morning molar tooth germ compared with P1-evening molar tooth germ

Expression of miRNAs in the two groups of mouse molar tooth germ was measured using microarray analysis as described in Materials and Methods. Based on the pattern of expression generated by the heat map (graphical representation of data where the values taken by a variable in a two-dimensional map are represented as colors), the miRNAs were divided in two groups (Group 1 and 2, as indicated on the left hand side). The colours indicate relative fluorescence intensities as shown on the right hand side. miRNA names are given on the right hand side. The heat map was generated using SPOTFIRE software.

Figure 4. Validation of microarray data using RT-PCR

The first mandibular tooth germs from two batches (P1-morning and P1-evening), each of five newborn pups, were dissected out and used to isolate total RNA and miRNA-enriched RNA fractions as described in Materials & Methods. Expression profiling of miRNAs and mRNAs was carried out using microarrays, and differentially expressed ($P<0.05$) miRNAs and mRNAs were isolated using the analysis of variance (ANOVA) function in the SPOTFIRE program. Gene expression was measured with microarrays and with real-time RT-PCR on 7 selected genes and 4 selected miRNAs. Plotted data represents mean ratios of level of expression at P1-morning / level of expression at P1-evening, with standard deviations indicated. Plotted data are derived from assays of five separate tooth germs in each experimental group. Experimental details are otherwise given in Materials and Methods.

The results of mRNAs (A) and miRNAs (B) are presented in the figure.

Figure 5. Top 10 cellular and molecular functions significantly associated with genes exhibiting *lower* levels of expression in the tooth germs from P1-morning compared with P1-evening mice

The genes were identified using the analysis of variance (ANOVA) function in the SPOTFIRE program, and subjected to bioinformatic analysis using Ingenuity Pathways Analysis (IPA). The plotted data represent associations between genes and cellular/molecular functional categories. The vertical line across the horizontal columns indicate the minimum value of -log(p), above which associations are judged to be significant as estimated by Fisher's exact test. The lengths of the horizontal columns indicate the level of significance computed for the various categories. Experimental details are given in Materials and Methods.

Figure 6. Top 10 cellular and molecular functions significantly associated with genes exhibiting *higher* levels of expression in the tooth germs from P1-morning compared with P1-evening mice

The genes were identified using the analysis of variance (ANOVA) function in the SPOTFIRE program, and subjected to bioinformatic analysis using Ingenuity Pathways Analysis (IPA). The plotted data represent associations between genes and cellular/molecular functional categories. The vertical line across the horizontal columns indicate the minimum value of -log(p), above which associations are judged to be significant as estimated by Fisher's exact test. The lengths of the horizontal columns indicate the level of significance computed for the various categories. Experimental details are given in Materials and Methods.

Figure 1

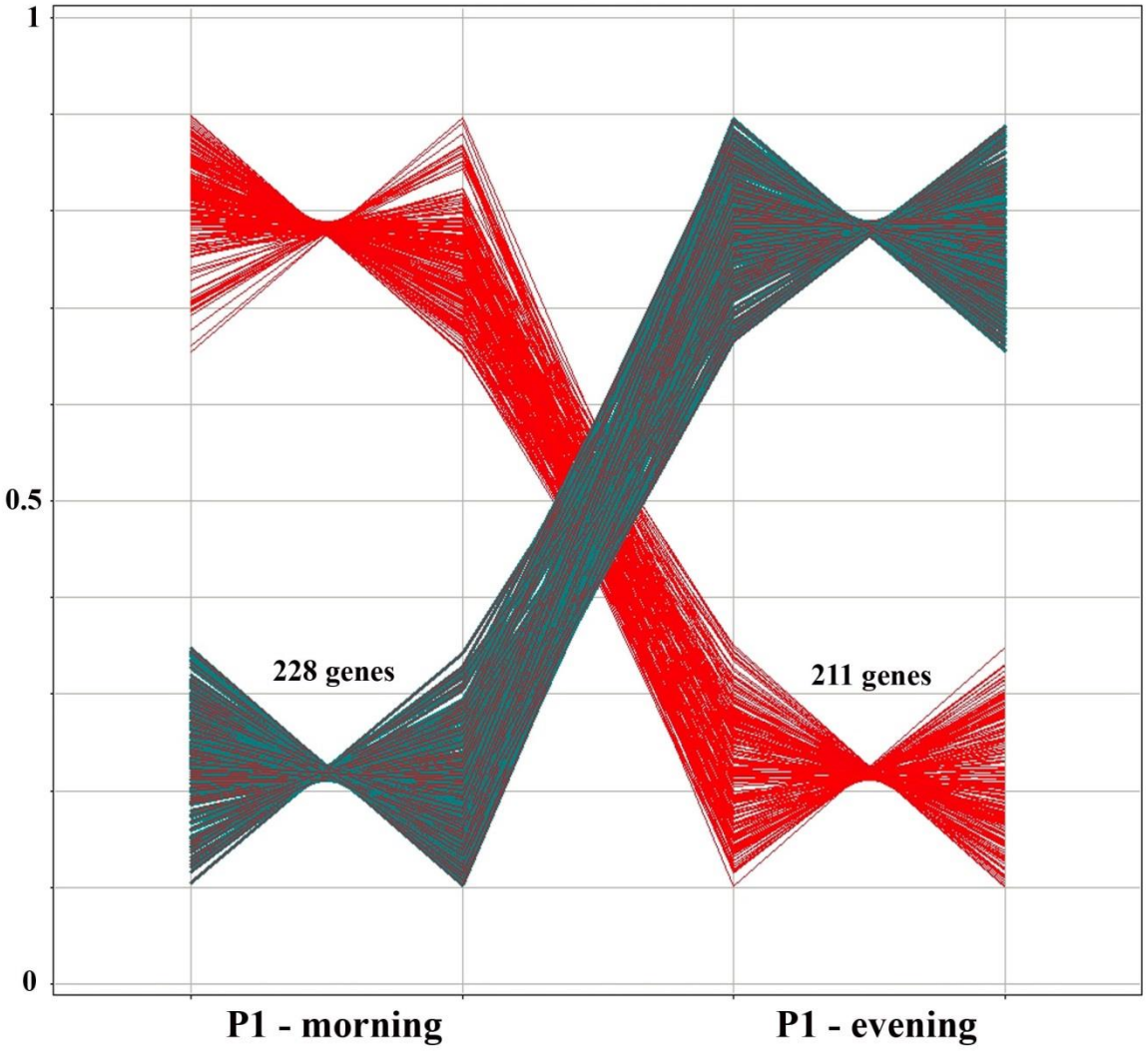


Figure 2

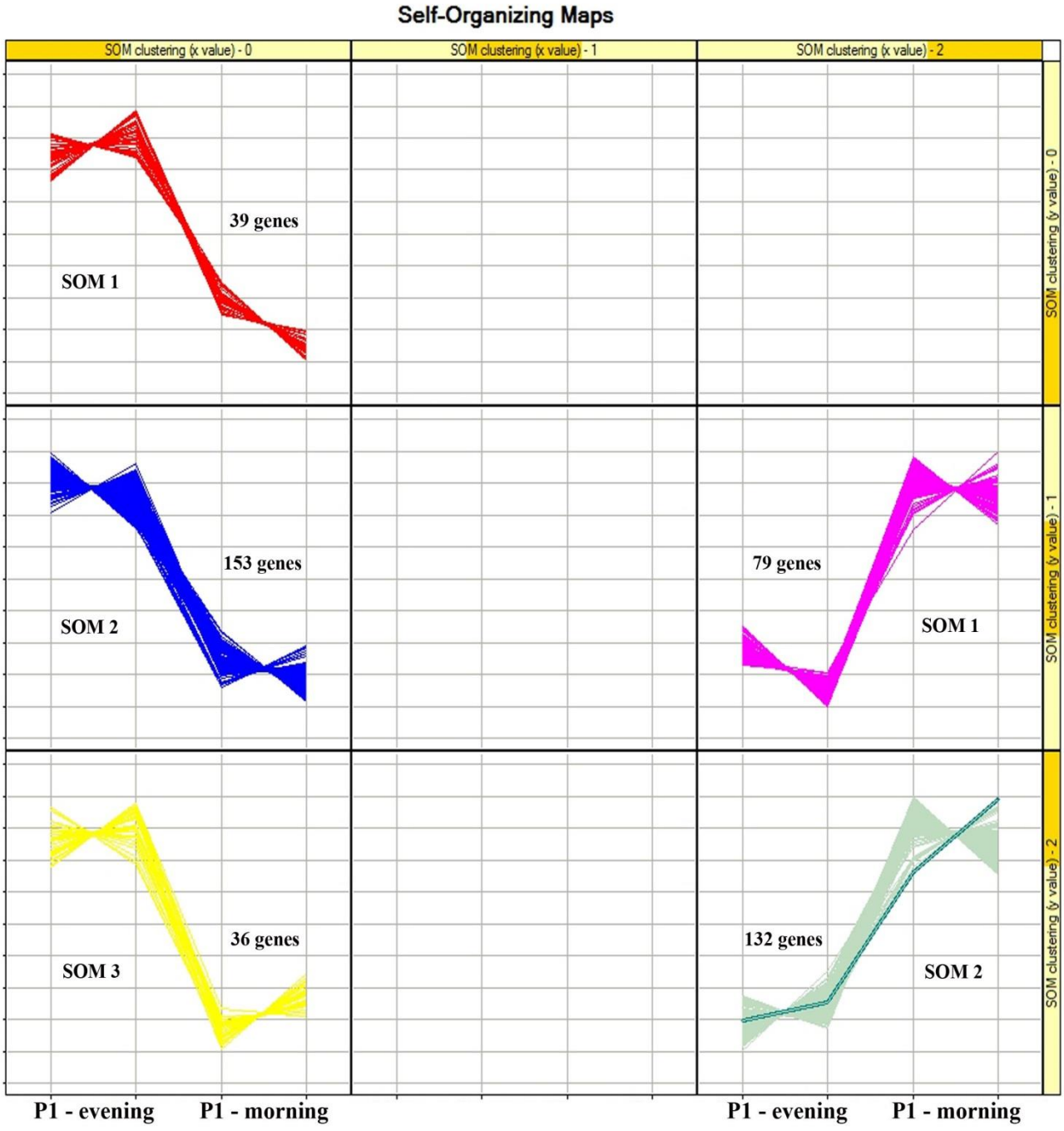

Self-Organizing Maps
SOM clustering (x value) - 0
SOM clustering (x value) - 1
SOM clustering (x value) - 2
SOM clustering (y value) - 0
SOM clustering (y value) - 1
SOM clustering (y value) - 2
39 genes
SOM 1
153 genes
SOM 2
79 genes
SOM 1
36 genes
SOM 3
132 genes
SOM 2
P1 - evening
P1 - morning
P1 - evening
P1 - morning

Figure 3

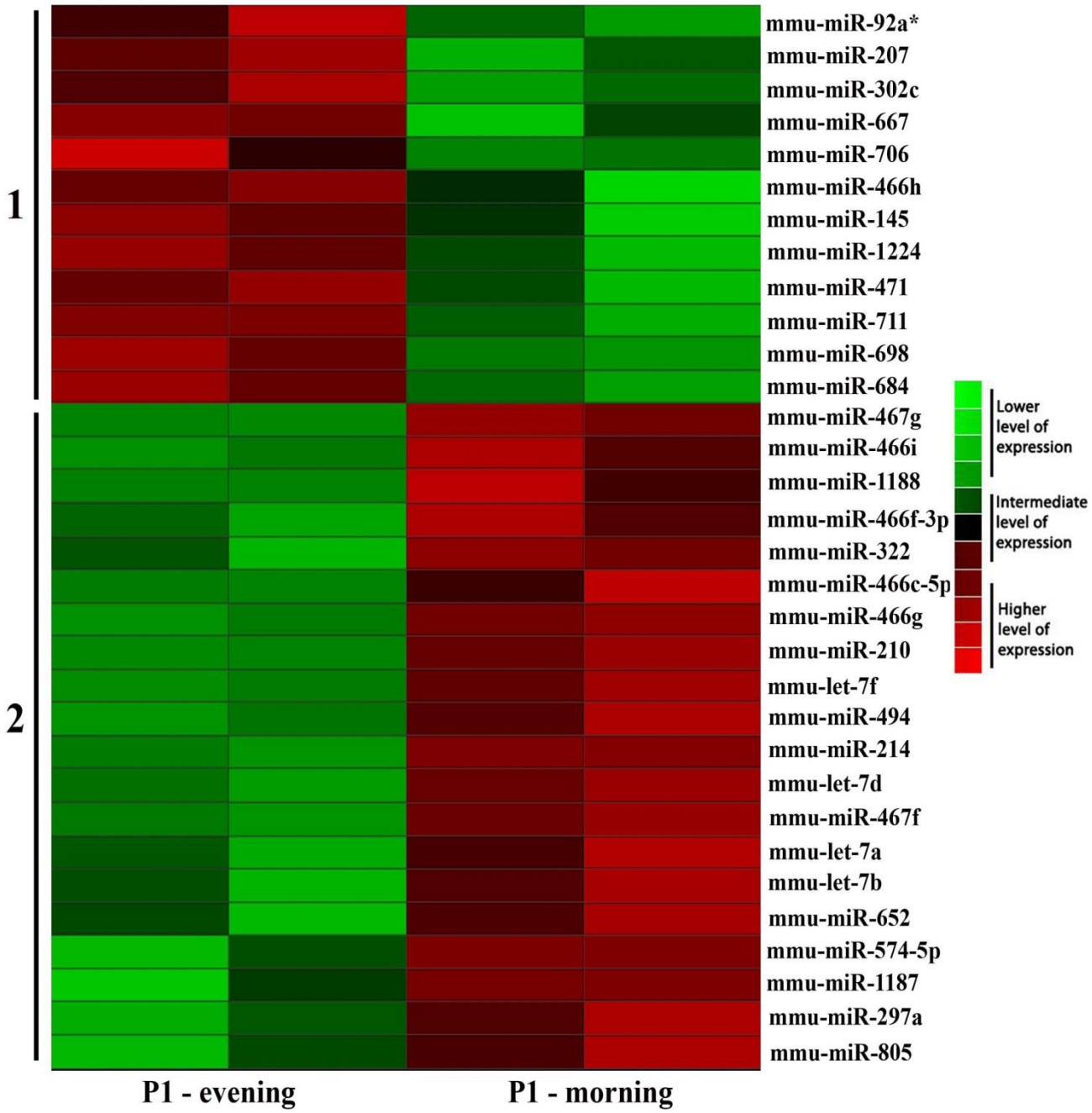

1
2
mmu-miR-92a*
mmu-miR-207
mmu-miR-302c
mmu-miR-667
mmu-miR-706
mmu-miR-466h
mmu-miR-145
mmu-miR-1224
mmu-miR-471
mmu-miR-711
mmu-miR-698
mmu-miR-684
mmu-miR-467g
mmu-miR-466i
mmu-miR-1188
mmu-miR-466f-3p
mmu-miR-322
mmu-miR-466c-5p
mmu-miR-466g
mmu-miR-210
mmu-let-7f
mmu-miR-494
mmu-miR-214
mmu-let-7d
mmu-miR-467f
mmu-let-7a
mmu-let-7b
mmu-miR-652
mmu-miR-574-5p
mmu-miR-1187
mmu-miR-297a
mmu-miR-805
Lower level of expression
Intermediate level of expression
Higher level of expression
P1 - evening
P1 - morning

Figure 4

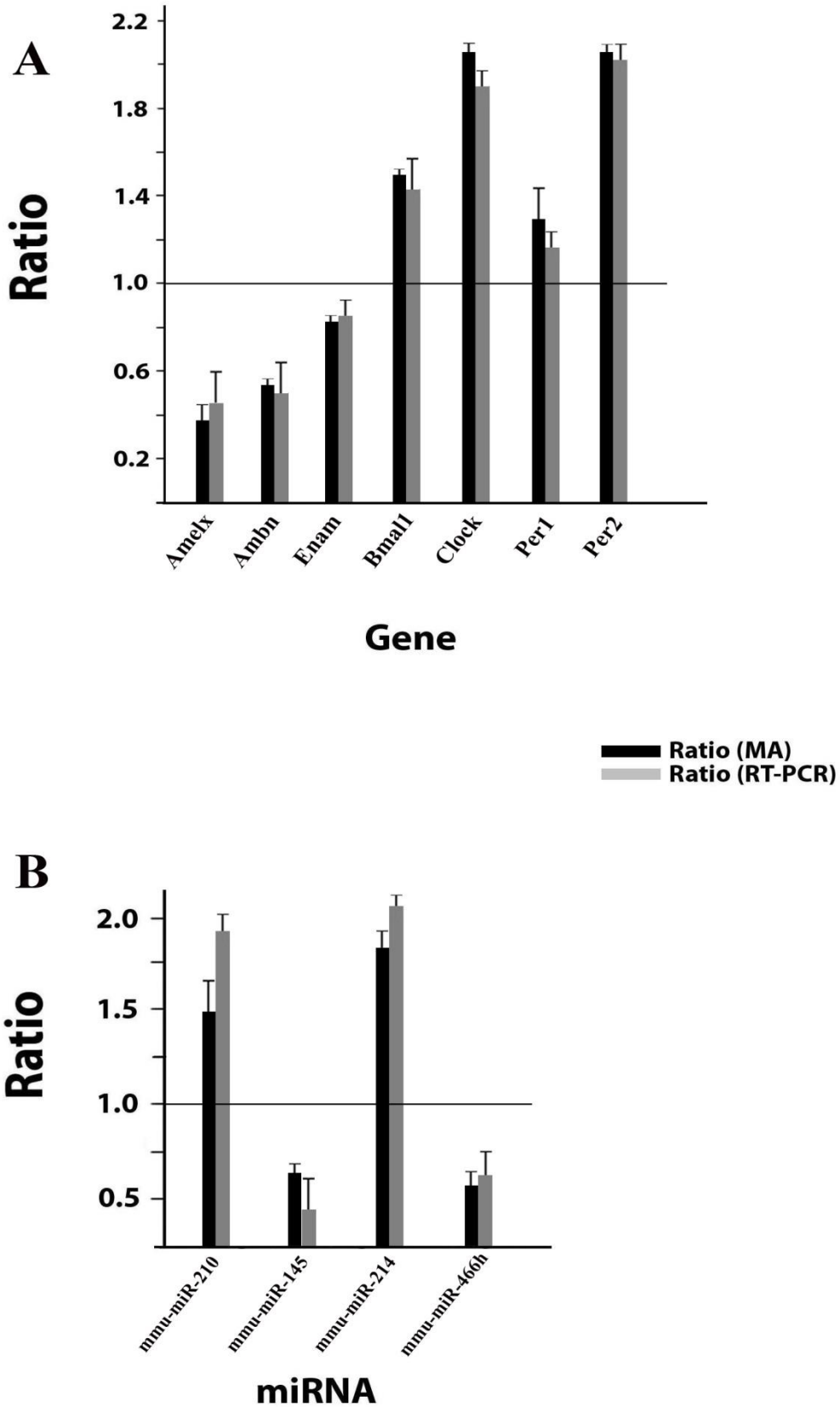

A
Ratio
2.2
1.8
1.4
1.0
0.6
0.2
Amelx
Ambn
Enam
Bmal1
Clock
Per1
Per2
Gene
Ratio (MA)
Ratio (RT-PCR)
B
Ratio
2.0
1.5
1.0
0.5
mmu-miR-210
mmu-miR-145
mmu-miR-214
mmu-miR-466h
miRNA

Figure 5

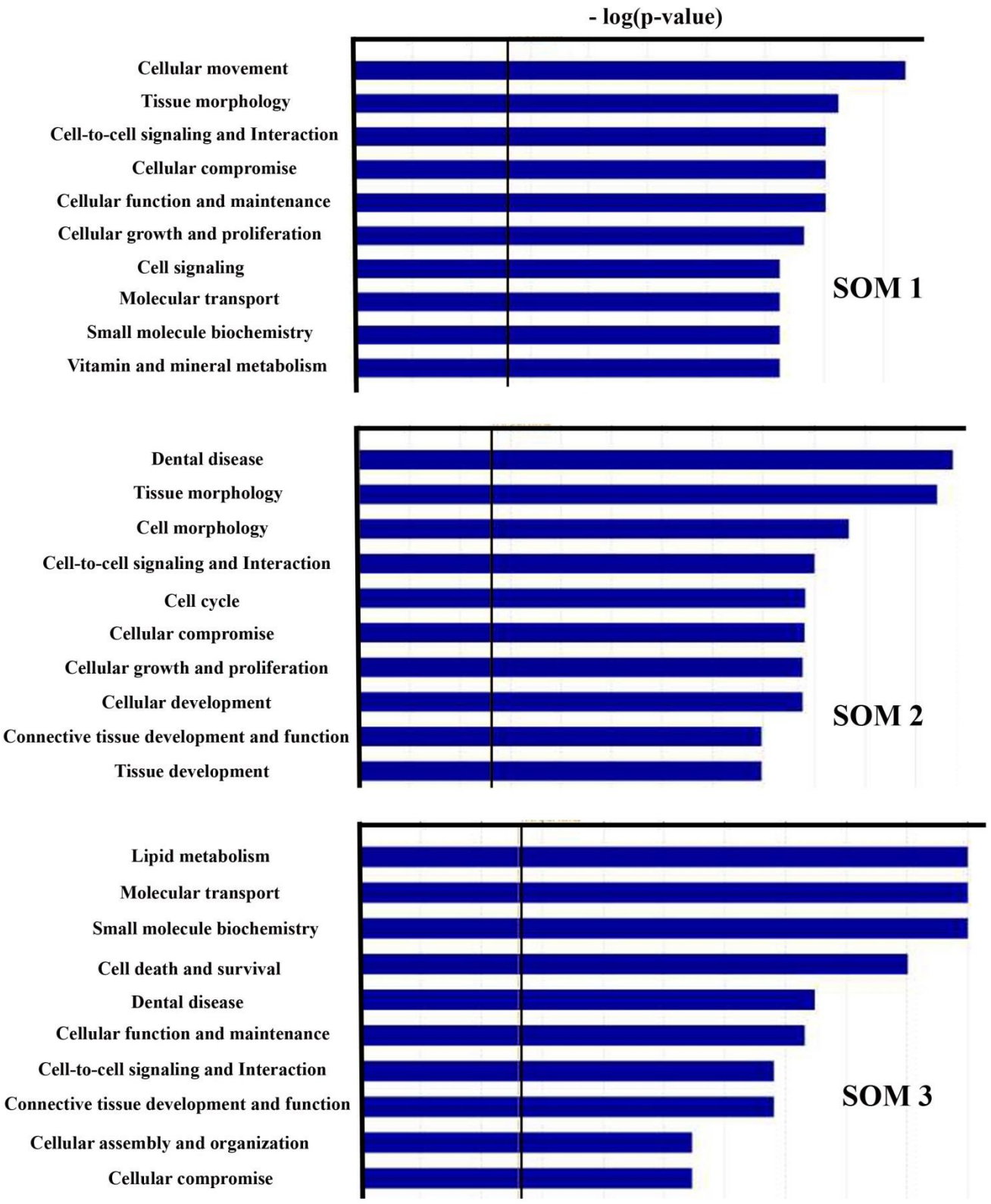
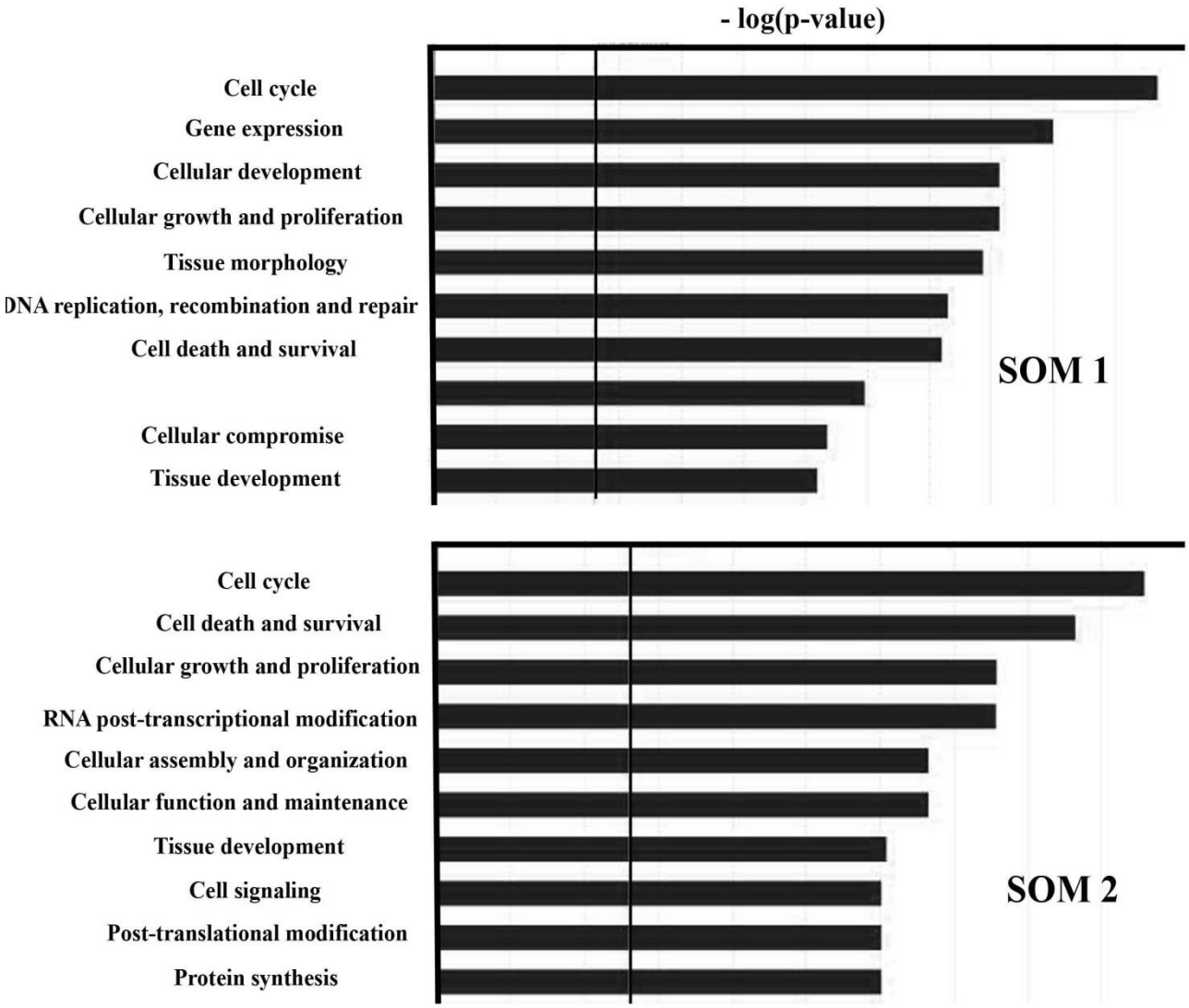

- log(p-value)
Cellular movement
Tissue morphology
Cell-to-cell signaling and Interaction
Cellular compromise
Cellular function and maintenance
Cellular growth and proliferation
Cell signaling
Molecular transport
Small molecule biochemistry
Vitamin and mineral metabolism
SOM 1
Dental disease
Tissue morphology
Cell morphology
Cell-to-cell signaling and Interaction
Cell cycle
Cellular compromise
Cellular growth and proliferation
Cellular development
Connective tissue development and function
Tissue development
SOM 2
Lipid metabolism
Molecular transport
Small molecule biochemistry
Cell death and survival
Dental disease
Cellular function and maintenance
Cell-to-cell signaling and Interaction
Connective tissue development and function
Cellular assembly and organization
Cellular compromise
SOM 3

Figure 6


- log(p-value)
Cell cycle
Gene expression
Cellular development
Cellular growth and proliferation
Tissue morphology
DNA replication, recombination and repair
Cell death and survival
Cellular compromise
Tissue development
SOM 1
Cell cycle
Cell death and survival
Cellular growth and proliferation
RNA post-transcriptional modification
Cellular assembly and organization
Cellular function and maintenance
Tissue development
Cell signaling
Post-translational modification
Protein synthesis
SOM 2

Legends to Tables

Table 1. Differentially expressed miRNAs and their mRNA targets

Predicted mRNA targets for differentially expressed miRNAs were identified using TargetScan and Ingenuity Pathway Analysis and are presented here. Each miRNA can target several genes/mRNAs. The first column (left) shows the miRNAs that exhibited different levels of expression in the P1-morning compared with P1-evening tooth germ. All the miRNAs are written without the prefix *mmu-miR*. miRNAs 7a, 7b, 7d, and 7f have the prefix *mmu-let.* The second column (middle) shows the genes that exhibited lower levels of expression at P1-morning, and the third column (right) shows the genes that exhibited higher levels of expression at P1-morning. Some of the miRNAs have similar sequences; these are annotated with a lower case letter. The results presented also indicate that some genes appear to be targeted by all the miRNAs which show similar sequences.

Table 2. Genes exhibiting lower levels of expression and their associated miRNAs

Table 2 presents 37 selected genes from SOM 1, 2 and 3 and show their associated miRNAs. The first column (left) presents genes that exhibited lower levels of expression at P1- morning. The second column shows miRNAs which are predicted to target the specific gene. All the miRNAs are written without the prefix *mmu-miR*. miRNAs 7a, 7b, 7d, and 7f have the prefix *mmu-let.* Some genes are targeted by several miRNAs, while some are not known to be targeted by any of the miRNAs we found to be differentially expressed in our data. Several of these genes are highly linked to tooth development. The predictions were performed using TargetScan and Ingenuity Pathway Analysis.

Table 1

miRNA	Genes with ***lower*** expression at P1-morning	Genes with ***higher*** expression at P1-morning
7a	*Fam20a, Pex26, Stxbp2, Odam, C7orf50*	*Igf2, Trip13, Pleckho1, Cdc25c, Parp1, Tgm2, Rbp1, Med4*
7b	*Fam20a, Pex26, Stxbp2, Odam, C7orf50*	*Igf2, Trip13, Pleckho1, Cdc25c, Parp1, Tgm2, Rbp1, Med4*
7d	*Pex26, C7orf50*	*Trip13, Pleckho1, Tgm2, Rbp1, Med4*
7f	*Pex26, C7orf50*	*Trip13, Pleckho1, Tgm2, Rbp1, Med4*
92a	*Tpcn1, Serpinf1, Muc5ac(Muc5b)*	*Mis18a, Adam19, Npnt, Myh3*
145	*Fam20a, Ror1, Papss2*	
207	*Enam, Rogdi(Lzf), Pex26*	*Ddx21*
210	*Tpcn1, Ror1*	
214	*Mrc2, Ctnna2(Catna2), Papss2, Muc5ac(Muc5b), Ltpb3, Fkbp9, Myoz1*	*Gart, Pcbp4, Tgm2, Rbp1, Ddx21, Pin4, Tmem48*
297a	*Papss2, Pex26*	
302c	*Ambn, Lama3, Enam, Odam*	
322	*Ctnna2(Catna2), Lnpep, Clu*	*Aurka*
466h		
466g	*Amelx, Rbm8a, Npc2*	*Incenp, Mad2l1, Tnnt, Ddx39b, Cryz, Tead2*
466i		
466f-3p	*Ambn, Amtn, Lum, Steap2, C1r*	*Incenp, Aurka, Ccnb2, Pus1, Nup93*
466c-5p	*LAMC2, CLU*	
467f	*Clk1, C1r, Pcdh7, Steap2*	*Cks2, Tacstd(Epcam), Incenp, Aurka, Pds5b,Pus1, Med4, Hist1h2ad*
467g	*Clk1, Fam20a, Pex26, Pcdh7, Hist1h2ad*	*Tacstd(Epcam), Mybpc1, Aurka, Cdc25c, Mis18a, Ccnb2, Htra1, Myh3, Nup93*
471		
494	*Ctnna2(Catna2), Papss2, Arsb, Fkbp9*	*Erh, Tgm2, Mrpl13*
574-5p	*Cacna1, Plac8*	*Erh, Mrpl13*
652	*Matn4*	
667	*Pex26*	
684	*Fkbp9*	*Aldh8a1, Med4, Psmb7*
698	*Odam*	
706	*Ambn*	*Trip13, Smpx, Cdc25c, Rbp1, Apip, Csrp3, Med4*
711	*Xnpep2, Pex26, C7orf50*	*Grb10, Myl1, Tubgcp2, Snrnp25, Myd88, Ddx21*
805		
1187	*Papss2, Pex26, Lum*	*Gemin5, Med4*
1188	*Fam20a, Pex26, Fkbp9*	*Myl1, Tead2, Gemin5*
1224	*Papss2, Pex26, Fkbp9*	*Cryz, Psmc1*

Table 2

Gene name	miRNA
C1r	467f, 466f-3p
Amelx	466g
Clk1	467f, 467g
Odam	302c, 7a, 7b
Tpcn1	92a, 210
Lnpep	322
Matn4	652
Xnpep2	711
Ambn	302c, 466f-3p, 706
Fam20a	7a, 7b, 145, 467g, 1188
Lama3	302c
Ror1	145
Lamc2	466c-5p
Enam	207, 302c, 494, 706, 1188
Mrc2	214
Ctnna2(Catna2)	214, 322, 494, 706
Amtn	466f-3p
Rogdi(Lzf)	207
Myoz1	214
Ltpb3	214
Fkbp9	214, 494, 684, 1188, 1224
Lamc2	466c-5p
Papss2	145, 214, 297a, 494, 1187, 1224
Rbm8a	466g
Pex26	7a, 7b, 7f, 7d, 207, 297a, 467g, 667, 711, 1187, 1188, 1224
C7orf50	7a, 7b, 7f, 7d, 711
Clu	322, 466c-5p
Pcdh7	467f, 467g
Lum	466f-3p, 1187
Steap2	466f-3p, 467f
Npc2	466g
Arsb	494
Cacna1	574-5p
Stxbp2	7a, 7b
Serpinf1	92a
Muc5ac(Muc5b)	92a, 214
Plac8	574-5p